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Review

Shooting at survivors: Bcl-2 family members as drug targets for cancer

Philippe Juin[a,*], Olivier Geneste[b], Eric Raimbaud[b], John A. Hickman[b]

[a] *Univ. de Nantes, INSERM U419, 44035 Nantes Cedex 035, France*

[b] *Institut de Recherche Servier, 125 chemin de Ronde, 78290 Croissy sur Seine, France*

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1. "Cancer is too complex to permit drug discovery"

A pessimistic view of the prospects for the discovery of selective chemotherapies for metastatic cancer would suggest that the molecular pathology of cancer is overwhelmingly complex. This pessimism stems from a variety of observations. Karyotype analysis of solid tumors, such as those of the colon, shows gross instability, with chromosomes in an apparently chaotic condition and the tumor in a state of permanent, unstable evolution [1]. Analysis of mutations in colon cancer, by the polymerase chain reaction, showed that there are upwards of 11,000 mutations in premalignant lesions [2,3]. And in a recent review, Loeb et al. [4], surveying the evidence that tumors have a "mutator phenotype", suggest that in a tumor of 10^8 cells, there are a billion different mutations. This presents a bleak outlook for a single, targeted therapy. It is thus considered that there is little chance to find one or two agents which, like antibiotics, could have a significant impact on such a complex, unstable disease. Moreover, those who suggest that each lesion, amongst tens or hundreds, should have a specific inhibitor targeted to it, do not seem to appreciate the practicalities of polypharmacy nor its economics, particularly with respect to clinical trials.

2. From thousands to five and from five to two

However, one may wonder whether the complexities of cancer—or indeed of biology itself—are as insoluble as they appear [5,6]. It has been suggested that the nature of biological or pathological complexity must be addressed by applying mathematical solutions, which have simplified complex problems in other fields [7]. Such sentiments permitted Reddy and Kaelin [8] to propose that a small number of targeted therapies are in fact a reality, since there are estimates that only five to ten causal mutations are necessary in the evolution of solid tumors [9], the rest being epiphenomenal changes. Indeed, recent experiments in transgenic mice support the view that metastatic tumors can be generated by a limited number of lesions. The results suggest that massive genomic instability is not necessary for tumor evolution and that progression from the initiating carcinogenic events to dependency on others is not necessary. Indeed, the idea that genomic instability is a key signature of cancer is hotly debated [10]. Thus, for tumor initiation and progression in the murine pancreas, Evan and colleagues showed, in ground-breaking experiments, that only *two* genetic changes needed to be engineered: the expression of a multifunctional oncogene (c-*myc*) and the inhibition of c-*myc*-induced apoptosis by targeted expression of the anti-apoptotic *Bcl*-X_L [11]. Subsequent suppression of c-*myc* expression in vivo, in animals with metastatic pancreatic tumors, resulted in complete tumor regression and the collapse of tumor neovasculature. Indeed, several similar murine models have shown that tumors in vivo, initiated by a single oncogene, remain "addicted" to that initiating oncogene and die by apoptosis when its expression is suppressed (reviewed in Refs. [12,13]). In "rewiring" the cell by expression of the initiating oncogene, clearly cell survival becomes compromised. Proliferation remains dependent on continued oncogene overexpression but necessarily in the context of a changed pattern of survival. This phenomenon of oncogene addiction, to the original oncogenic event, was independent of the character of the initiating onco-

* Corresponding author. Tel.: +33-2-4008-4083; fax: +33-2-4008-4082.

E-mail address: pjuin@nantes.inserm.fr (P. Juin).

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gene: in vivo tumors were created using switchable c-*myc* or *ras* or Bcr-Abl, and all were ''cured'' of their tumors by suppression of oncogene expression.

3. The interplay of apoptosis and proliferation and environment in cancer pathogenesis

There is now a well-defined coupling between cell proliferation and cell death. Indeed, most mechanisms leading to cell proliferation, including deregulated expression of c-Jun, deregulated expression of c-Myc and virtually any pathway that would lead to the activation of the transcription factors of the E2F family have also the ability to promote apoptosis [14]. Thus, a mitogenic lesion endangers the affected cells, as exemplified by the spectacular apoptosis that accompanies c-Myc activation in pancreatic β cells [11]. A cell whose proliferation is deregulated has to escape from apoptosis in order to expand and form a tumor. An anti-apoptotic ''platform'' is thus fundamental to tumor initiation, progression and maintenance. A great number of cancer cells exhibit anti-apoptotic mutations (see below). An expanding cancer cell population has also to modify its surrounding milieu, as it is replicating outside of its normally adequate environment which provides anti-apoptotic signals. These signals normally come from soluble factors, physical interactions with the neighbouring cells or with the extracellular matrix. Moreover, tumor progression is accompanied by a certain number of stress signals that promote apoptosis, such as nutrient deprivation, hypoxia or telomere erosion. Not surprisingly, therefore, evasion of apoptosis is a fundamental hallmark of cancer [15].

Understandably, the fact that cancer cells are under strong selective pressure to evade apoptosis casts doubt on the use of empirical apoptotic triggers in order to eradicate them [16]. Anti-apoptotic lesions that can allow a neoplastic cell to survive also render cancer cells resistant to many classical cancer therapeutics, whose goal is mainly to produce an apoptotic response [17]. Because cancer cells are continuously under the pro-apoptotic influence of both their deregulated proliferation and ectopic growth, they are inherently sensitized to apoptosis despite their acquired pro-survival lesions. As a result, they may be acutely dependent upon those mutations to survive: shrewd inhibition of an anti-apoptotic pathway may thus be potently harmful to a cancer cell, while hardly affecting a normal cell safely protected by its trophic niche. This justifies new therapeutic strategies aiming to target the aberrant mechanism of survival of cancer cells. In that respect, a better characterization of the molecular pathways of apoptosis, and of the defects in such pathways that cancer cells exhibit, will lead not only to efficient, but also to specific, anti-cancer therapy: the pathology itself is used as the ''driver'' for cell death. The key question arises as to how many survival pathways and proteins might need to be targeted by drugs?

4. The Bcl-2 family of proteins are at an apical point of the apoptotic machinery

Strategies which target either survival signaling or the activation of tumor suppressor activity all act at some point of diversity in the cell death pathway. Therefore, their success can be strongly hampered by defects in the apoptotic machinery itself. The Bcl-2 family members that regulate apoptosis and mitochondrial integrity [18] are at the apex of the decisional ''life or death'' tree, and present a limited number of key targets for therapeutic intervention. Among the Bcl-2 family members, the pro-apoptotic Bax and Bak display sequence conservation throughout three Bcl-2 homology domains (BH1 to 3). Once they are in an active conformation, these multi-domain pro-apoptotic proteins are intrinsically cytolytic. Both have the innate ability to perturb the membrane permeability of mitochondria where they localize. This results in the release of numerous apoptogenic proteins (such as, for instance, cytochrome *c*) from the mitochondrial intermembrane space, by a mechanism that is still debated [19,20]. In addition, Bax and Bak may control apoptosis at the endoplasmic reticulum (ER), where they also reside, by regulating ER Ca^{2+}-dependent apoptotic stimuli [21].

The deleterious effects of Bax/Bak are counterbalanced by the anti-apoptotic activity of survival members such as Bcl-2, Bcl-xL or Mcl-1 that display all four Bcl-2 homology domains (BH1 to 4) and the ratio between anti-apoptotic and multi-domain pro-apoptotic Bcl-2 family members helps determine the cellular susceptibility to death stimulation [22]. Anti-apoptotic Bcl-2 family members localize at the mitochondria, where they can prevent the release of apoptogenic factors that otherwise occurs in response to apoptotic stimuli. They also reside at the ER, and they could play a role in regulating ER Ca^{2+} dynamics [23]. The protective function of Bcl-2/Bcl-xL appears to be, in great part, built upon their faculty to form inactivating heterodimers with Bax/Bak [24]. As discussed below, these proteins may nevertheless exert some of their survival effects independently from this sequestering function.

A third subgroup of pro-apoptotic Bcl-2 family members display sequence homology only with the BH3 domain [25]. These BH3-only proteins bind to, and functionally antagonize, Bcl-2/Bcl-xL via their eponymous homology domain. They seem to act as the afferent effectors of various pro-apoptotic signals, integrating them into one single death pathway mainly mediated by the multi-domain proapoptotic Bax and Bak [26–28]. Ectopic expression of either Bcl-2 or Bcl-xL, or the combined genetic ablation of both Bax and Bak, renders cells resistant to a great variety of apoptotic stimuli, unambiguously showing that the Bcl-2 family members guard a necessary gateway to apoptosis.

An imbalance among the Bcl-2 family of proteins, in favor of the anti-apoptotic members, is a phenomenon that naturally, and frequently, occurs in cancer cells. Indeed, overexpression of anti-apoptotic Bcl-2 or Bcl-xL probably

occurs in more than half of all cancers. Moreover, loss of expression of Bax is also found in some colorectal cancers and in hematopoietic malignancies [29,30], whereas the expression of a highly apoptogenic variant of Bax is correlated with an increased survival of glioblastoma multiforme patients [31]. Those defects may arise, at least in part, from the fact that neoplastic cells are under strong selective pressure to stabilize their mitochondrial permeability, even if they harbor alterations in the p53 tumor surveillance pathway. c-Myc, for instance, can induce mitochondrial damage independently from the transcriptional activity of p53 [32,33]. Since p53 also activates the mitochondrial death pathway, the mitochondrion appears to integrate the diverse pro-apoptotic mechanisms induced by oncogenes. This integrative role is a key feature promoting the molecules as potential therapeutic targets. Studies in transgenic mice have indeed revealed that Bcl-2 (and/or Bcl-xL) overexpression and p53 mutations (or ARF loss) are selected for independently during Myc-induced lymphomagenesis [34].

Unfortunately, such defects will also hinder the efficiency of most therapies that aim at inducing apoptosis in cancer cells, as they all rely on a Bcl-2-dependent pathway to exert their deleterious effects (even including those based on the stimulation of the death receptor pathway: see Ref. [35] and references therein). Thus, new strategies need to be developed in order to tackle the cytoprotective activity of Bcl-2/Bcl-xL.

5. The therapeutic strategies

5.1. Gene therapy and antisense approaches

One current approach uses gene therapy-based delivery of Bax [36,37]. Recombinant adenoviruses encoding for Bax are highly toxic, even to healthy cells (see Ref. [36] and references therein). This approach therefore requires optimization as expression of the transgene has to be selectively targeted to transformed cells. This can be achieved with the use of a recombinant adenovirus in which Bax expression is driven by the human telomerase reverse transcriptase promoter. This adenovirus was shown to induce Bax expression selectively in cancer cells (and thus to diminish the secondary effects on healthy cells) and to eradicate tumor growth in xenograft models [38].

The use of nuclease resistant antisense oligonucleotides targeting the Bcl-2 mRNA has reached to Phase III clinical trials for melanoma, myeloma, acute myeloid leukaemia and chronic lymphatic leukaemia [39]. Experiments in vitro and in mouse models have shown that BCL-2 antisense increases the sensitivity to conventional chemotherapeutics, and phase II data indicate that the combination of the antisense approach and of chemotherapy may increase therapeutic success. Other antisense-based strategies have been developed. These include the use of either BCL-XL, or MCL-1 antisense oligonucleotides [40], the use of BCL-X antisense oligonucleotides designed to shift pre-mRNA splicing away from producing the anti-apoptotic Bcl-xL protein to the proapoptotic one Bcl-xS [41] or that of BCL-2/BCL-XL bispecific antisense oligonucleotides [42]. Even if some of these approaches were promisingly shown to induce apoptosis in some tumor cells, or to sensitize them to chemotherapy in vitro, there is still some uncertainty as to which, if any, of these anti-apoptotic proteins (that are frequently co-expressed in cancer cells) is the most important survival factor for one given tumor type. This challenging question is complicated by the fact that some of the Bcl-2 family members may exert, in cancer cells, functions that differ from their anti-apoptotic one. Bcl-2 and Bcl-xL have both been found to regulate the cell cycle by a mechanism that is still debated [43,44]. Thus, antisense oligonucleotides may have an unwanted proliferative effect in certain cells [45]. Along this line, it is also important to note that high Bcl-2 expression correlates with good prognosis in breast cancers (see Ref. [46] and references therein). Although the mechanism involved in this paradoxical effect of Bcl-2 is yet to be characterized, it indicates, to the very least, that down-regulating certain anti-apoptotic Bcl-2 family members may not always be appropriate.

5.2. Targeting Bcl-2 and Bcl-X_L with peptides

An alternative approach to counter the protective effects of Bcl-2/Bcl-xL is to try to directly antagonize some of the survival functions of these proteins. The three-dimensional analysis of Bcl-xL [47] and Bcl-2 [48] has revealed an hydrophobic pocket at the surface of these proteins, formed by their BH1, -2 and -3 domains. These domains are all necessary for the survival function of the anti-apoptotic Bcl-2 members, and the hydrophobic groove they form plays an important role in their inhibitory binding to pro-apoptotic counterparts. In particular, it appears that this site serves as a recipient for the conserved BH3 domain of BH3-only proteins. This suggests that synthetic peptides encompassing minimal BH3 domains may be used as a basis to develop functional antagonists of Bcl-2/Bcl-xL, which would prevent the sequestration of pro-apoptotic members by these survival proteins.

Experiments using either unmodified, microinjected, peptides or peptides attached to cell permeant moieties of either lipidic [49] or peptidic [50] origin have shown promising results with respect to cancer therapy. First, peptides indeed bind to survival proteins of the Bcl-2 family [47], antagonize their protective activity against death-receptor or DNA-damage induced apoptosis [50,51] and are cytolytic per se [49,51,52]. Second, c-Myc activation in primary murine cells increases their sensitivity to apoptosis induction by BH3 peptides [51]. The molecular mechanism involved remains to be characterized, and it would also be important to know whether this effect extends to other mitogenic lesions. This suggests, nevertheless, that cancer cells may be intrinsically sensitized to the deleterious effects

of Bcl-2/Bcl-xL antagonists and may explain the remarkable ability of some cell permeant BH3 peptides to induce apoptosis in human myeloid leukemia in vitro, slowing their growth in immunodeficient mice, while having little effect on normal peripheral blood lymphocytes [49]. Third, the ability of BH3 peptides, and their ability to cooperate with c-Myc, is intact in p53-deficient cells [51]. Thus, in cells harboring a deficient p53 pathway, peptidic antagonists of Bcl-2/Bcl-xL represent an alternative to the aforementioned antisense approach, which is essentially used in combination with classical chemotherapy, and whose efficiency may be altered by p53 deficiency [53]. Fourth, specific induction of apoptosis by BH3 peptides requires the presence of either one of the two multi-domain pro-apoptotic proteins Bax or Bak [51]. This is also of potential interest, as cancer cells that exhibit a loss in Bax expression usually retain Bak expression. The sole loss of Bax may thus significantly lower their apoptotic response, but recruitment of Bak activity by the use of a small BH3 peptide may restore sensitivity to apoptosis [51].

At the very least, these observations constitute a proof of concept validating strategies based on generating molecules that occupy the BH3 binding pocket of Bcl-2/Bcl-xL. As all the binding information in BH3 domains is derived from one short contiguous sequence, one possible approach is to design mimetics of the peptide inhibitors. There is no such peptidomimetic described so far, but the modified molecules should exhibit increased cell permeability and increased stability when compared to the peptides they are derived from, which are poorly cell permeant per se and, presumably, highly unstable given their short α-helical structure. Moreover, the helical structure of BH3 peptides may also be troublesome, as α-helices can often be nonselectively toxic to cells: mutant BH3 peptides that lack the ability to bind to Bcl-2/Bcl-xL exhibit a weak, nonspecific cytotoxicity [51]. Although this modest effect can be easily differentiated from the stronger effects specifically elicited by wild type BH3 peptides in model experiments, it may limit the usefulness of BH3-peptides in vivo. One alternative approach to overcome this possible problem is to discover small-molecule, nonpeptide Bcl-2/Bcl-xL antagonists.

5.2.1. Small molecule antagonists of Bcl-2 and Bcl-X_L

To date, more than a dozen compounds that may antagonize Bcl-2/Bcl-xL have been described (reviewed recently in Refs. [54–56]). A selection of such small molecule inhibitors of Bcl-2 or Bcl-X_L is shown in Fig. 1A.

Among these, some have been initially discovered from in silico screens based on the predicted structures of the BH3 binding site of either Bcl-2 or Bcl-xL [57,58]. The

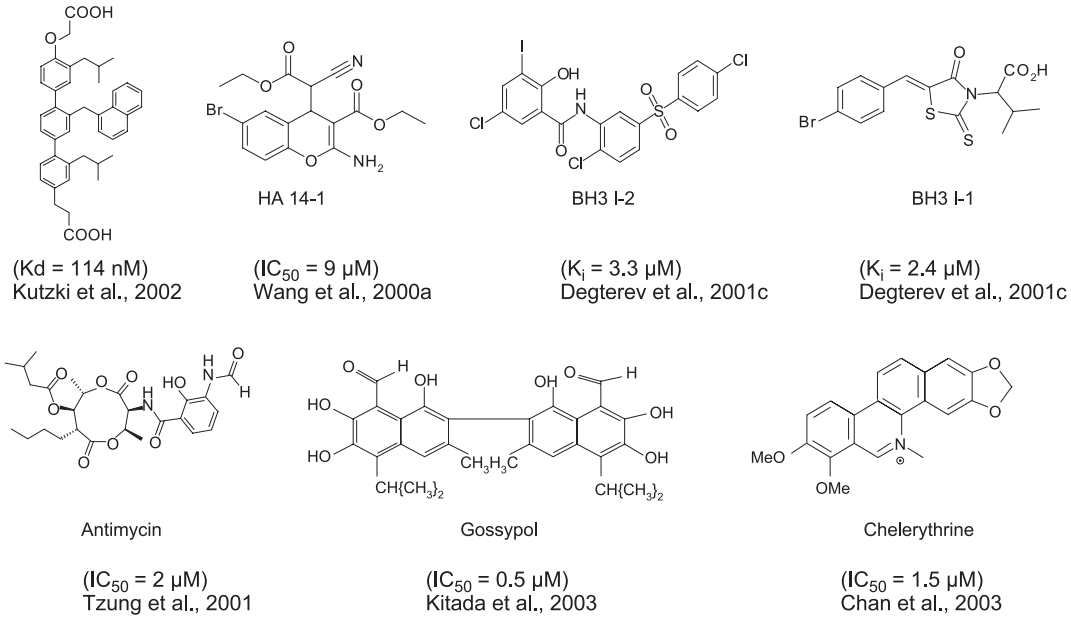

Fig. 1. Small molecule inhibitors of Bcl-2/Bcl-xL. (A) Structure of some of the compounds discovered so far. (B) Molecular models of ligands bound to Bcl-xL. The optimized structure of Bcl-xL (pdb 1BXL) is represented by its Connolly Surface. The entire protein is shown except for the disordered C-terminal histidines region. The surface formed by residues from the conserved BH1, BH2 and BH3 domains are colour-coded in yellow, red and green, respectively. The rest of the surface is in white. The hydrophobic cleft is formed by residues from BH1, BH2 and BH3 domains. The apoptotic ligands are represented by stick models; the carbon, oxygen, nitrogen, sulfur and halogens are colour-coded in white, red, blue, yellow and green, respectively. a, complex with Antimycin A1; b, complex with HA-14-1; c, complex with BH3-i1; d, complex with BH3-i2; e, NMR original complex with the BH3 domain of the Bak protein (1BXL non-optimized). All structures were modeled in SYBYL® 6.9. All the hydrogen atoms were added on the NMR structure pdb1BXL of the protein and their geometries were optimized using the Tripos force field, using Kollman charges on all atom of the protein and Gasteiger–Hückel charges on the ligand atoms. Each compound was then docked manually in this binding cavity and its conformation was fully optimized with Tripos force field, while the whole protein was kept fixed in aggregate, except the side chains of the amino acid residues of the groove in close contact with the ligands which were allowed to change their conformations during the optimization. TRIPOS ASSOCIATES Inc., 1699 S. Hanley Road, Saint Louis, Missouri 63144, USA.

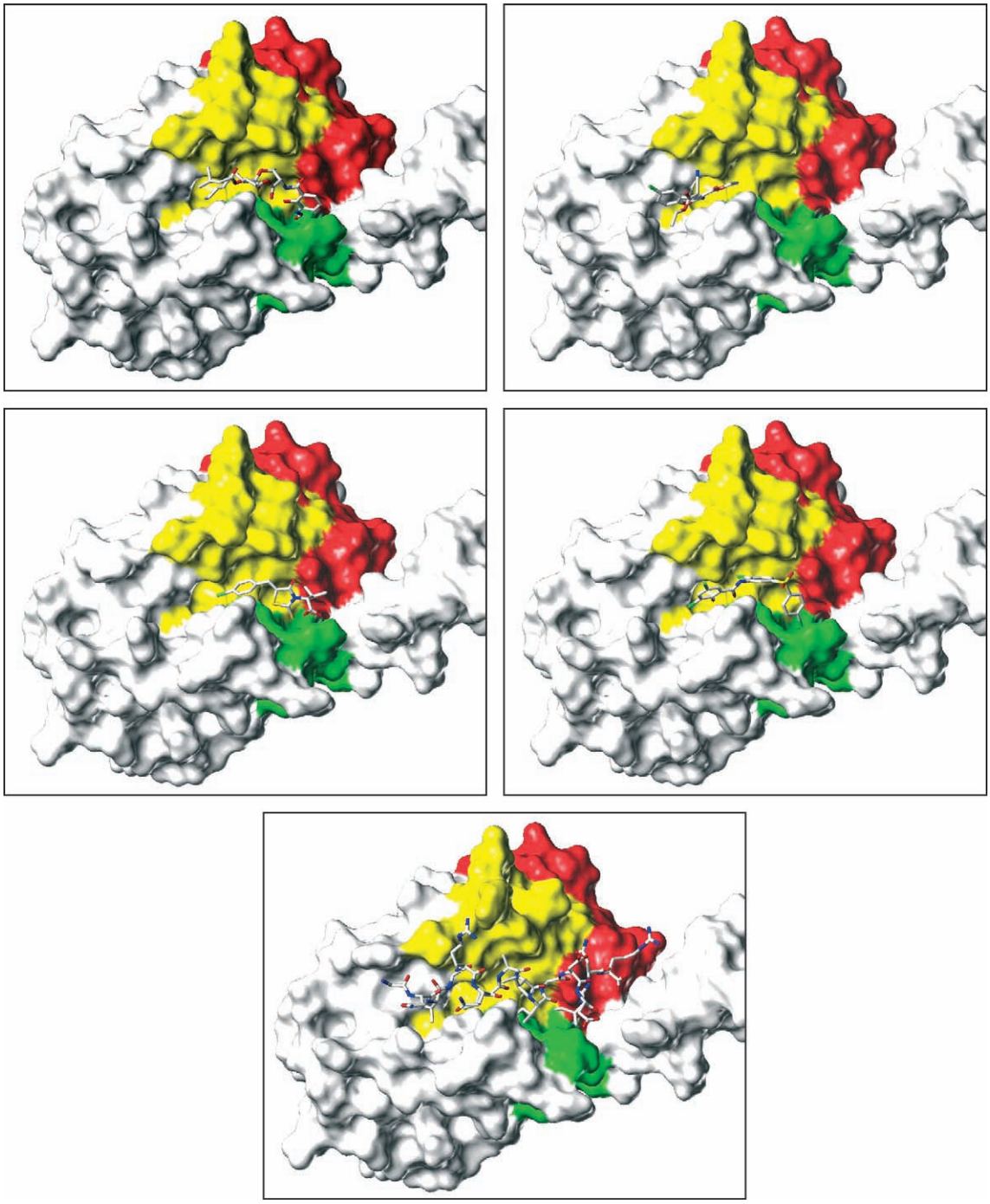

Fig. 1 (*continued*).

ability of the small organic compound HA14-1 to prevent binding of a BH3 peptide to Bcl-2 was monitored in a polarization fluorescence assay [57]. Moreover, this compound selectively induces apoptosis in malignant hematopoietic cell lines that overexpress Bcl-2 [59], in agreement with the notion that it acts as a direct inhibitor of this protein. It has been proposed, moreover, that Bax expression is a key determinant of its cytotoxic effect [60]. Enyedy et al. [58] have characterized other small compounds that also prevent binding of Bcl-2 to a BH3 peptide, bind directly to Bcl-xL as assayed by NMR and selectively induce apoptosis in cancer cells with high levels of Bcl-2.

Other compounds were found in a more straightforward screen for molecules that can disrupt the complex between Bcl-xL and a BakBH3 peptide. Degterev et al. [61] have identified by this approach novel small molecules, BH3Is, that, as revealed by NMR studies, can occupy the BH3 binding domain of Bcl-xL, disrupting interactions between

the latter and its pro-apoptotic counterparts in vivo, and inducing apoptosis. BH3Is molecules have also been used in a ligand optimization method that combines structural and computational approaches and that has led to the discovery of other potential inhibitors [62]. A polarization fluorescence-based assay similar to the one used by Degterev and coworkers has also been used in order to screen for natural products. Kitada et al. [63] have characterized certain polyphenols as such inhibitors. Gossypol and Purpurogallin were shown to bind to the hydrophobic pocket of Bcl-xL by heteronuclear NMR spectroscopy techniques. The former exhibits the ability to induce apoptosis, even in Bcl-xL overexpressing cells, whereas the latter is much less efficient, presumably as a consequence of its hydrophylic character. Chan et al. [64] have also characterized chelerythrin, a compound previously known as a protein kinase C inhibitor, as a compound that can inhibit binding of Bcl-xL to either a BH3 peptide or full-length Bax and that can induce apoptosis in cells overexpressing Bcl-xL by directly triggering mitochondrial permeabilization.

Antimycin A, an inhibitor a respiration, was fortuitously characterized as a Bcl-xL inhibitor through its ability to induce apoptosis in Bcl-xL overexpressing cells [65]. Further studies have shown that this compound, or a derivative thereof that lacks the ability to inhibit respiration, prevents the binding of a BH3 peptide to recombinant Bcl-2 and induces mitochondrial permeabilization.

All the aforementioned molecules were discovered by approaches that focus on the structure, or the function, of the BH3 binding site of anti-apoptotic Bcl-2 family members. No obvious structural consensus arises from the highly diverse molecules discovered so far (see Fig. 1A), and it also appears that their respective binding to the hydrophobic pocket of their target may involve distinct structures (see Fig. 1B). All of these molecules nevertheless seem to recapitulate some of the functional effects of BH3 peptides, in as much as they can bind to either Bcl-xL or Bcl-2, disrupt heterodimers these form with pro-apoptotic counterparts, and induce apoptosis. Antimycin A was shown to bind to both Bcl-xL [65] and Bcl-2 [66], despite the structural differences in the respective surface pockets of these proteins [48]. It is, however, unclear whether compounds that were specifically selected for their ability to bind one anti-apoptotic Bcl-2 member will also antagonize the others. Most of the current molecules exhibit a disappointingly low affinity for their targets, thus raising doubts on the specificity with which they induce apoptosis. However, a report of a molecular scaffold, mimicking the alpha-helix of the BH3 domain of Bak, has provided an inhibitor with a K_D of 114 nM, measured in a Bak peptide-Bcl-X_L binding assay [67]. These types of molecules are templates for drug design, and modifications improving their biological activity are required before the balance between their anti-tumor activity and their side effects against normal cells can be evaluated.

5.3. Mechanistic questions—how well do we understand the dynamics of Bcl-2 protein family interactions?

Even though BH3 mimetics with high efficiency could be developed using peptides or small molecules as lead compounds, there are still some urgent issues that need to be addressed. The first issue stems from our relatively poor understanding of how BH3-only proteins promote apoptosis. Evolving evidence indicates that these proteins, as well as BH3 peptides, induce apoptosis by a mechanism that requires the presence of either Bax of Bak. In viable cells, these multi-domain proteins appear as inactive proteins residing at the mitochondria (Bak) or in the cytosol (Bax) [68]: they need to undergo a change in their conformation to exert their apoptotic function. Thus, in order to induce Bax/Bak dependant apoptosis, a certain signal has to activate these proteins in some way. Certain BH3 peptides seem to have the ability to directly activate those proteins and allow their pro-apoptotic assembly in the mitochondrial membrane [69]. It is therefore possible that certain BH3 mimetics may have the ability to directly activate Bax/Bak and function as death agonists (Fig. 2). It has to be stressed, however, that occupancy of the surface pocket of the Bcl-2 family members and ligand-induced activation of Bax/Bak seem to display distinct structural requirements: some other BH3 peptides [69] or even small molecules antagonists of Bcl-2/Bcl-xL [70] seem to lack the ability to activate Bax/Bak. As the latter compounds nevertheless induce apoptosis, it seems important to understand whether, and how, such survival antagonists recruit Bax/Bak activity by inhibiting Bcl-2/Bcl-xL. One possibility is that they can release from their anti-apoptotic targets some intermediate death agonists that, in turn, lead to Bax/Bak activation (Fig. 2). It should be noted, however, that despite its inability to self-associate, Bax was shown to interact with Bcl-xL in model experiments [71]. Peptidic antagonists of Bcl-xL, moreover, release from such complexes active forms of Bax. Therefore, survival antagonists may promote the pro-apoptotic assembly of activated Bax without any other intermediaries than Bcl-2/Bcl-xL themselves (Fig. 2), by a mechanism in which, most intriguingly, anti-apoptotic proteins would cooperate with their antagonists to induce apoptosis [71].

Along this line, the fact that Bcl-2 and Bcx-L overexpressing cells are highly *sensitized* to apoptosis induction by antimycin A [65,66] is striking. It will be of interest to know whether other small molecule inhibitors of Bcl-2/Bcl-xL recapitulate this effect, but it is obvious that deciphering the mechanism involved may lead to the development of attractive strategies that are selectively efficient against cancer cells that overexpress Bcl-2 or Bcl-xL. One straightforward approach towards the characterization of the mode of action of any given molecule may be the identification of the proteins, in one given cell type, that this compound releases from Bcl-2/Bcl-xL. It should be noted, moreover, that posttranslational modifications, such as that induced by proteolytic cleavage, can convert Bcl-2 or Bcl-xL into

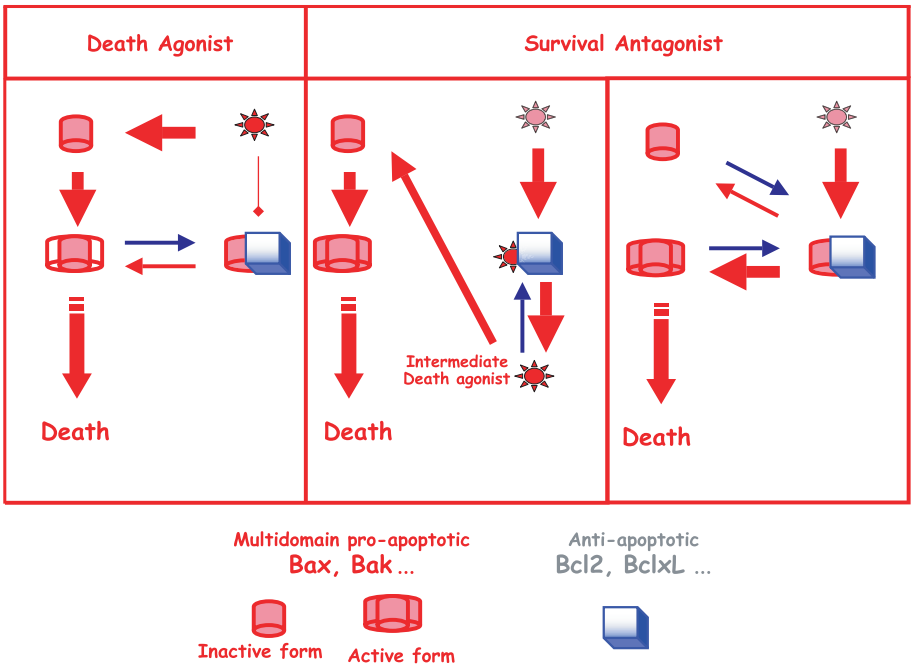

Fig. 2. Modes of activation of the pro-apoptotic multi-domain proteins Bax/Bak by BH3-like molecules. Certain BH3 mimetics may function, like the BH3 domain of Bid, as "death agonists" (red star) that directly trigger the activation of Bax and Bak (A). Anti-apoptotic proteins interfere with this process by inhibiting activated Bax/Bak or by directly binding to the death agonists. Other BH3-like molecules may, in a similar manner as the BH3 domain of Bad, function as "survival antagonists" that only interact with Bcl-2/Bcl-xL (pink stars). They may induce apoptosis by provoking the release from Bcl-2/Bcl-xL of an endogenous death agonist (red star in B) that in turn activates Bax/Bak, as proposed in Ref. [28]. Alternatively (C), such survival antagonists may promote apoptosis without the involvement of an intermediate agonist, by directly releasing an active Bax from heterodimers with anti-apoptotic proteins [71]. See text for further details.

potent pro-apoptotic proteins [72,73]. It is therefore formally possible that certain ligands of Bcl-2/Bcl-xL may sufficiently alter the conformation of these proteins to turn them into pro-apoptotic effectors, even though there is, to date, no evidence for such putative effect.

5.4. Possible pitfalls

It is uncertain whether induction of apoptosis by Bcl-2 antagonists will be efficient in all cancer cells. If such antagonists function as true BH3 mimetics, then changes in the conformation of Bax (and of Bak), its relocation from cytosol to mitochondria and its oligomerization in the mitochondrial membrane should be important for the initiation of apoptosis by such molecules. However, recent evidence indicates that none of these changes are alone sufficient to commit a taxol-resistant neuroblastoma cell line to apoptosis [74]. The defect that these cells display is yet to be characterized, but it appears to result in limited mitochondrial damage and cytochrome *c* release in response to an apoptotic stimulus. The fact that the stores of cytochrome *c* are heavily subcompartimentalized within healthy mitochondria has led to suggest that a profound remodelling of mitochondria is involved in the complete cytochrome *c* release observed in apoptotic cells (see Ref. [75] and references therein). Surprisingly, the BH3 only protein Bid induces dramatic changes in the inner membrane shape of mitochondria that could participate in the complete release of cytochrome *c* that this protein triggers. This effect is independent from the BH3 domain of Bid and does not require Bax and Bak, even though these two proteins are necessary for Bid to induce full apoptosis (see Ref. [76] and references therein). Other candidates for the reorganization of mitochondrial structure that occurs during apoptosis are proteins that physiologically shape the mitochondrial reticulum, as exemplified by the finding that a dominant negative form of Drp-1, a dynamin family protein normally involved in mitochondrial fission, interferes with apoptosis induction [77]. Defects in these ill-characterized mitochondrial remodelling pathways may strongly hamper the efficiency with which Bcl-2 inhibitors promote apoptosis.

Another issue is raised by the complex relationship between pro-apoptotic Bcl-2 family members and caspases. Genetic ablation of caspases (caspase 9 and caspase 3), which act downstream of cytochrome *c* release, seems insufficient to promote resistance to cell death induction by ectopic expression of BH3-only proteins [28]. Moreover, the broad spectrum inhibitor of caspases zVADfmk had no significant effect on the onset of apoptosis and cytochrome *c* release induced by microinjected BH3 peptides [51], while significantly extending the execution phase of apoptosis (as assayed by time lapse video-microscopy). However, recent evidence, using specific silencing of other individual caspases [78] or a more potent caspase inhibitor [79], indicates

that some caspases may function upstream of the mitochondrial death pathway. One implication from these observations is that, although some caspases may be dispensable for the cytotoxic effects of BH3 mimetics, others may play an essential role in the efficiency with which they promote apoptosis. Many cancer cells overexpress inhibitors of apoptosis protein (IAPs) that can endogenously quell the activity of some caspases. Silencing of some IAPs by specific antisense oligonucleotides has been shown to induce apoptosis in some cancer cells, or to sensitize them to chemotherapy, indicating that overexpressed IAPs may significantly impact on the cellular response to apoptosis. IAPs can be functionally antagonised by small peptides derived from a tetrapeptidic motif in Smac that binds to the BIR domains of IAPs. Although Smac is considered to function as a dimer [80], such monomeric Smac peptides exhibited a remarkable ability to sensitize various tumor cells, both in vitro and in vivo, to apoptosis induced by death receptor ligation or chemotherapy. They bypass the survival effects of Bcl-2 or xIAP without significantly altering normal cells [35]. Smac agonists are thus promising cancer therapeutics that may be extremely useful when combined with a cancer-specific cytotoxic therapy. It will be of great interest, in particular, to evaluate their functional interaction with Bcl-2 antagonists in resistant tumors.

Most strikingly, Bcl-2 appears to suppress the caspase activation program that was reported to function upstream of mitochondria by Marsden et al. [79]. Another implication from this is that Bcl-2 may be directly involved in protein–protein interactions with a "caspasomal" complex, pretty much in the same way as its *C. elegans* equivalent, CED9, interacts with the scaffolding protein CED4. A CED4-like equivalent that could bind to Bcl-2 is yet to be characterized in mammals, but, if it exists, its displacement from Bcl-2 may play a key role in apoptosis induction. As a result, the ability of various BH3 mimetics to disrupt complexes between Bcl-2 and this unknown CED4-like protein (in a similar manner as the *C. elegans* BH3-only protein Egl-1 does) could be one major parameter. Along this line, it is also important to note that Bcl-2/Bcl-xL has been reported to interact with numerous proteins that do not contain a BH3 domain (such as R-Ras, Raf-1, calcineurin; reviewed in Ref. [81]). Thus, although the potent pro-apoptotic activity of BH3-mimetics (see above) suggests that sequestering BH3-containing proteins is one major way by which Bcl-2/Bcl-xL protects cells from apoptosis, other functions of Bcl-2/Bcl-xL may not be abrogated by certain BH3 mimetics.

Finally, the question of the degree of in vivo selectivity possible for molecules targeted to perturb Bcl-2/Bcl-xL function may depend not only on the extra requirements of a tumor to support its survival via these molecules. It may also depend on the role that Bcl-2/Bcl-xL plays in maintaining not only normal cell survival but other, homeostatic, functions of the cell. Recent work has shown that Bcl-xL may play a role in controlling the synaptic response and stability of certain neurons by facilitating ATP flux across the mitochondrial membrane [82]. Thus, strategies targeted to alter Bcl-2/Bcl-xL function may have secondary effects outside the domain of apoptosis.

6. Conclusions

The recent and spectacular success of the tyrosine kinase inhibitor Glivec® in the clinic has confirmed that novel drugs can be effective and selective if targeted to a molecular lesion implicated in the pathology. In the case of Glivec®-sensitive chronic myelogenous leukemia, this is the Bcr-Abl oncogene. Is targeting a defined single molecular lesion going to be possible in colon cancer? This is debatable. We suggest here that targeting the survival platform of tumor cells, a platform that underpins their ability to carry mutations, genetic instability and aberrant oncogene expression, may be tumor selective. With the Bcl-2 family of proteins at the pinnacle of the "decision network" for death and survival (as the finding that the double knockout of the pro-apoptotic proteins Bax and Bak renders cells pleotropically resistant to stress, suggests), there is also hope that the number of drug targets will be limited, while selectivity would come from the inherent differences between tumor and normal cells, reflected in their load of genomic damage and inappropriate expression of oncogenes. Using the general features of the molecular pathology of cancer as the driver of cell death is indeed attractive. Like all hopes for a more selective therapy of cancer, time and biology will be the arbiters.

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